NEW TESTS FOR CHOLINE IN PHYSIOLOGICAL FLUIDS.

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In view of the importance of the presence of choline in the cerebro-spinal fluid and blood in cases of degenerative disease in the nervous system, as demonstrated by the researches of Mott and Halliburton, Donath, and others, it seemed desirable to examine critically the principal chemical test (that with platinum chloride) which has been used for the detection of this substance.

At Prof. Halliburton's suggestion, I therefore undertook an investigation of the solubilities of the chlorides of potassium, sodium, and ammonium in absolute alcohol, and of their platinum salts in 15 per cent. alcohol under the conditions employed in the platinum test for choline. In the course of this work, I came to the conclusion that these solubilities, although very small, were nevertheless such as to make, under unfavourable conditions, a contamination of the final choline platino-chloride with platino-chlorides of potassium or ammonium, a possible source of error. It is difficult to distinguish the crystals of these substances microscopically, and the application of other tests like the determination of melting point is excluded owing to the small quantities of material usually obtained. It was therefore necessary to search for other tests for choline, which are not given by salts of potassium or ammonium.

Three tests have been the outcome of this research. They may be briefly termed—

1. The per-iodide test; this is a microscopic method.

2. The alloxan test; a colour reaction.

3. The bismuth test; this depends on the formation of a characteristic precipitate.

1. *The Per-iodide Test.* If a strong solution of iodine in potassium iodide[1] is added to a preparation of choline platinum chloride crystals, a most characteristic change takes place which can be watched under the microscope. In the course of a minute or so, the yellow octahedral or prismatic crystals of the platino-chloride disappear, and their place is taken by dark brown plates and prisms in large quantities. Their growth may be watched, and they obtain sometimes a length of 0·8 mm. so that they can then be seen with the naked eye. They show marked dichroism (green and brown), and they have a superficial resemblance to the well-known hæmin or Teichmann's crystals. They are doubly refracting and appear light-brown in the dark field of the polarising microscope with crossed Nicols. If the slide is allowed to stand, so that the liquid gradually evaporates, the crystals begin to disappear, and their place is taken by brown oily droplets, or sometimes clear spaces showing the skeletons of the crystals are seen. If now a fresh drop of the iodine solution is added, the crystals gradually form once more, and their formation and disappearance may be watched repeatedly.

From the manner of their formation and disappearance, it seems that we have here to deal with an unstable per-iodide of choline[2]. The action of potassium iodide on the platinum salt produces first of all the soluble iodide of choline; this enters into combination with excess of iodine to form the insoluble per-iodide crystals. On exposure to air this compound dissociates, the iodine evaporates, and soluble choline iodide is left, which will again form the per-iodide on the addition of more iodine.

The platino-chlorides of ammonium and of potassium remain unchanged under the action of the iodine solution.

The important point of the test as above described is that it is applied to the platino-chloride. The reaction occurs, however, with the other salts of choline. It seemed therefore feasible to apply it directly to the residue of the alcoholic extract of physiological fluids. It was found, nevertheless, that the presence of other substances in this residue

[1] The composition of the solution which was found to answer well was: 2 g. iodine and 6 g. potassium iodide dissolved in 100 c.c. water.

[2] This crystalline substance seems to have been first observed by Griess and Harrow (*Ber. d. Deutsch. chem. Ges.* XVIII. p. 717, 1885) who used it for the isolation of choline from hops and beer. In 1896 Florence (*Arch. d'anthropol. crimin.* X. XI.) published a test depending on the formation of such crystals on the addition of iodine to semen. He thought it was characteristic of human semen. It was shown, however, by Bocarius (*Zeitsch. f. physiol. Chem.* XXXIV. p. 339) that they owe their origin to the presence of choline.

rendered the test somewhat uncertain. It is therefore best to isolate the platino-chloride first; and for this purpose I followed the method described by Halliburton and Mott[1], except that the use of really absolute alcohol is not essential. The precipitate obtained by adding platinum chloride is dissolved in a few c.c. of 15 per cent. alcohol, and this solution after filtration is placed upon glass slides and evaporated at 40° C. Under such circumstances crystals of the platino-chloride of choline are found mixed with those of potassium and ammonium. On the addition of the iodine solution, the latter remain unchanged, but the crystals of the choline salt even when present in small quantities undergo the characteristic change already described[2].

It has been possible to detect choline in 20 c.c. of blood, when the choline has been added in the proportion 1 : 20,000. When 5 c.c. of blood containing choline in the proportion 1 : 2000 are used, the whole field is covered with crystals, which can be seen with the naked eye.

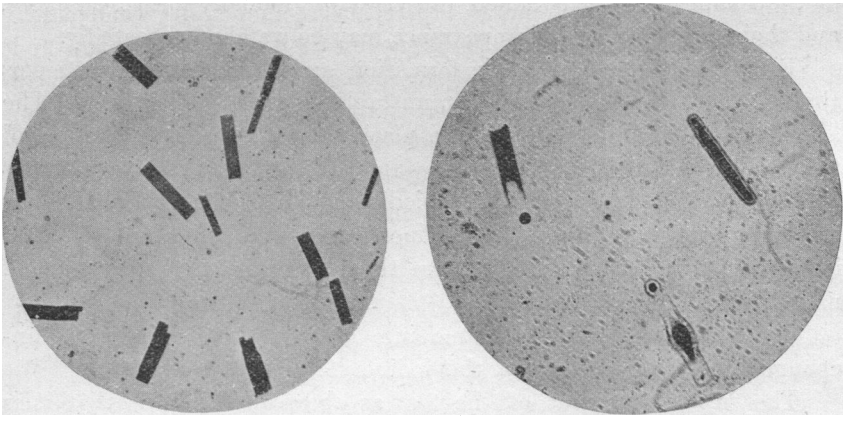

Fig. I. is a microphotograph of the crystals of choline per-iodide. Fig. II. shows their disintegration and the formation of oily droplets.

2. *The Alloxan Test.* All the usual reagents employed to obtain colour reactions with alkaloids fail when applied to choline. It was, however, observed that a drop of 1 per cent. solution of choline hydrochloride, to which one or two drops of a saturated solution of alloxan (Kahlbaum) were added, gave on evaporation in the water-bath a beautiful rose-violet colour which changed to a deep blue-violet on

[1] *Phil. Trans. Roy. Soc.* CXCI. 1899; *Ibid.* CXCIV. 1901.

[2] The platinum salts of other organic bases show similar changes, and yield characteristic crystals which differ from those of choline per-iodide. I intend to further investigate the reaction for the investigation of physiologically important bases.

the addition of sodium or potassium hydrate. Mineral acids discharge the colour. The reaction resembles the well-known murexide test for uric acid. Proteids in solution or in the dry state, as well as ammonium salts, also give the reaction; but potassium or sodium salts do not.

It can also be obtained from the choline platino-chloride, by adding to a concentrated solution of this salt a few drops of a concentrated solution of potassium chloride. The heavy precipitate of potassium platino-chloride so formed is filtered off or allowed to settle. In the latter case the supernatant fluid, in the former case the filtrate, is evaporated on the water-bath in a white porcelain basin after the addition of a few drops of a saturated solution of alloxan. The colour reaction then appears.

The test may be applied to the detection of choline in cerebro-spinal fluid or blood by getting rid of proteids and other substances in the usual way, and then isolating the platinum salt. It is, however, essential that the latter should be free from ammonium compounds. This can easily be done by a slight modification of Halliburton and Mott's method. The residue of the first alcoholic extract is taken up with acidulated water; lime water is then added until the reaction is slightly alkaline. This liquid is left standing over dilute acid, for the absorption of the liberated ammonia, or evaporated slowly on a water-bath. By this treatment all the ammonium salts are decomposed, whilst the choline present is not attacked. This was demonstrated by quantitative experiments in which known amounts of choline were added. The further treatment in the preparation of the platinum salt and application of the colour reaction is the same as before. The degree of sensitiveness of this reaction is still being investigated.

3. *The Bismuth Test.* Very little attention has been paid to the reaction of choline with potassium bismuth iodide since the introduction of this reagent by Dragendorff in 1866[1]. This is no doubt due to the small degree of sensitiveness the reagent is supposed to show with choline[2]. Dragendorff's reagent contains a large excess of potassium iodide, and when the test is carried out according to his directions in a sulphuric acid solution, hydriodic acid is set free, and this exerts a solvent action on the precipitate. The delicacy of the reaction is enormously increased when a solution containing only the necessary amount of potassium iodide is used. I accordingly adopted the mode

[1] *Jahresb. f. Chem.* p. 1821. 1866.

[2] Compare Gulewitsch, *Zeitsch. f. physiol. Chem.* xxiv. p. 519.

of preparation of the reagent given by Kraut[1]; this solution has the further advantage of keeping well. A drop of this solution added to 1 or 2 c.c. of a very dilute solution of choline gives at once a characteristic brick-red, amorphous precipitate; Dragendorff's reagent fails to do this or only produces the dirty brownish precipitate which it gives with distilled water alone. Kraut's reagent also produces a brownish precipitate with distilled water, but in the presence of minute quantities of choline the bright brick-red precipitate is formed. The presence of potassium or ammonium salts does not seem to have the slightest influence. Proteids, however, are also precipitated by this reagent, and have therefore to be removed. For this purpose the physiological fluid to be tested is extracted with absolute alcohol, and this treatment is repeated several times with the residue of the extraction. The final residue is taken up with 2 or 3 c.c. of water, and then a drop of the reagent added. The limit of sensitiveness of the reaction has not yet been investigated, but a strong reaction was still obtained with the extract of 20 c.c. of blood containing choline in the proportion 20,000 : 1.

In a future paper I hope to publish my results with these tests when applied to the detection of choline in cases of disease.

Since this note was written v. Staněk has published a paper on the per-iodide of choline (*Zeitsch. f. physiol. Chemie*, XLVI. p. 280) in which he describes the crystals formed by the interaction of choline and iodine solution. He studied the conditions under which they are formed, isolated and analysed them and found that they possess the composition of a choline-ennea-iodide of the formula $C_5H_{14}NOI.I_8$. He has also worked out a method for the quantitative estimation of choline based on the formation of this compound.

The expenses of this research have been defrayed from a grant made to me by the Government Committee of the Royal Society.

[1] *Ann. d. Chem.* CCX. p. 310. (A solution of 80 g. bismuth-subnitrate in 200 g. nitric acid (*spec. grav.* 1·18) is poured into a concentrated solution of 272 g. potassium iodide in water. Potassium nitrate crystallises out and is filtered off. The filtrate is diluted with water to 1 litre.)